FEBS Letters 455 (1999) 276–280 FEBS 22351

Murine leukemia provirus-mediated activation of the *Notch1* gene leads to induction of *HES-1* in a mouse T lymphoma cell line, DL-3

Jong-Seo Lee[a], Akinori Ishimoto[a], Tasuku Honjo[b], Shin-ichi Yanagawa[a,*]

[a]*Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*
[b]*Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*

Received 28 April 1999; received in revised form 21 June 1999

Abstract Constitutive activation of Notch signaling is known to be associated with tumorigenesis. In a mouse T lymphoma cell line, DL-3, we found that a murine leukemia provirus was inserted in the *Notch1* locus, which led to marked expression of a virus-Notch1 fusion mRNA encoding an intracellular portion of the Notch1 protein. Furthermore, expression and nuclear localization of this constitutively active form of Notch1 protein were confirmed. Corresponding to this finding, the transcription of the *hairy/enhancer of split* (*HES-1*) gene, a known target of Notch1 signaling, was elevated in this cell line. A potential role for overexpressed HES-1 in the development of the lymphoma was discussed.

© 1999 Federation of European Biochemical Societies.

Key words: Notch1; HES-1; Insertional mutagenesis; Murine leukemia virus; Lymphoma

1. Introduction

Notch family genes encode transmembrane receptor proteins mediating signals which regulate various cell fate decisions that involve cell-cell interactions [1]. The Notch proteins are characterized by an extracellular domain containing EGF-like repeats which bind the Delta-Serrate-Lag2 family of ligand proteins, and Notch/lin-12 repeats of unknown function, and by an intracellular domain containing a RAM domain and Cdc10/ankyrin repeats, both of which can bind the RBP-J/CBF-1 transcription factor [1–3]. Recent reports showed that the binding of Notch ligand induces proteolytic cleavage of Notch [4–10]. The cleaved intracellular form of Notch makes a complex with RBP-J/CBF-1, translocates into the nucleus and regulates transcription of target genes, such as the *hairy/enhancer of split (HES)* gene encoding a basic helix-loop-helix transcription factor [10–13].

Loss of the extracellular domain of the Notch protein causes constitutive activation of the protein, known to be associated with induction of lymphomas [14–17] and mammary tumors [18]. In human T cell acute lymphoblastic leukemia, the chromosomal translocation t(7;9) joins a portion of Notch1/Tan1 to the T cell receptor β locus [14]. The *Notch1* gene is also known to be a target of provirus insertions in T cell lymphomas arising in Moloney murine leukemia virus (Mo-MuLV)-infected MMTV^{D}/myc transgenic mice suggesting that c-*myc* and *Notch1* collaborate for oncogenesis [15]. Furthermore, Robey et al. have demonstrated that the overexpression of the activated form of mouse Notch1 biases T cell development toward CD8 single-positive T cells, indicating that Notch1 is involved in T cell fate determination [19]. However, the target(s) of Notch1 signaling in the processes of T cell development and transformation remains elusive.

Here, we describe a mouse T lymphoma cell line, DL-3, with a MuLV provirus insertion in one of the Notch1 alleles, which led to ectopic expression of a truncated Notch1 mRNA and protein. The transcription of*HES-1*, a member of the *HES* family, was markedly elevated in this cell line, suggesting that the leukemogenic function of the truncated Notch1 protein was mediated by activation of *HES-1*.

2. Materials and methods

2.1. Cell culture

The three T lymphoma cell lines DL-3, 333A, and DL-5 were established from lymphomas spontaneously developed in DBA/2 mice in our laboratory [20] and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

2.2.. Southern and Northern blot analyses

Twenty micrograms of genomic DNA extracted from lymphoma cell lines was digested with restriction endonucleases, and Southern blotted as described [21]. For Northern blot analyses, 10 μg of total RNA was prepared and analyzed as described [21].

The following DNA fragments were isolated from the pBluescript II KS containing the *Notch1* cDNA and used as probes for Southern and Northern analyses: middle probe, a 0.7-kb *Bam*HI-*Sac*I fragment encoding the region from the C-terminus of the EGF-like repeats to the N-terminus of the transmembrane domain. Extracellular probe: a 2.8-kb *Cla*I fragment encoding the region from the N-terminus to EGF-like repeats. Intracellular probe: a 1.9-kb *Not*I fragment encoding the region from the Cdc10/ankyrin repeats to the C-terminus of Notch1. A 1.3-kb *Eco*RI fragment from pSV2-CMV HES-1, the plasmid containing rat *HES-1* cDNA (a gift from Dr. R. Kageyama, Kyoto University) was used as a probe for Northern analysis. A 3.5-kb *Bam*HI fragment from pPAN7, the plasmid containing human elongation factor-1α cDNA (a gift from Dr. S. Nagata, Osaka University) was used as a probe for RNA loading control. For quantitative analysis of Northern blotting, the X-ray films were scanned with a densitometer (Shimadzu, Japan).

2.3. Screening and sequencing of Notch1 cDNA

The cDNA library of DL-3 was prepared from poly-(A)$^{+}$ RNAs selected with the Poly(A) Quick mRNA Isolation Kit (Stratagene), by using the ZAP Express cDNA Synthesis Kit and ZAP Express GigapackII Gold Cloning Kit (Stratagene). To obtain clones with the *Notch1* sequences, the cDNA library was screened with a ^{32}P-labeled *Notch1* intracellular probe. The clones hybridized with intracellular probe were rescued as pBK-CMV plasmids by in vivo excisions and the cDNA inserts in these plasmids were sequenced. Thermo Sequenase (Amersham) Kit and 373 SEQUENCER (Applied Biosystems) were used for nucleotide sequence analysis. Sequence homology between the cDNA insert and the *MuLV LTR* ([22], GenBank accession number M13079) or the *Notch1* cDNA ([2], GenBank accession number Z11886) was analyzed. In this report, all nucleotide positions of the *Notch1* cDNA and *MuLV* are cited from these databases. The nucleotide sequences of the regions of interest of the clone dl-3B

*Corresponding author. Fax: (81) (75) 751-3995.
E-mail: syanagaw@virus.kyoto-u.ac.jp

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)00901-1

(see Fig. 2A) have been entered in EMBL Nucleotide Sequence Database with the accession number AJ238029.

2.4. Immunoblot analysis

The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, and Western blotted as described [23]. The blot was incubated with rabbit antibody against the RAM23 domain of the Notch1 protein, followed by the peroxidase-conjugated secondary antibody against rabbit IgG (Bio-Rad). The blots were visualized with the Enhanced Chemiluminescence Reagent (Amersham).

2.5. Immunostaining

DL-3 cells were fixed, permeabilized, and blocked as described [23]. The samples were processed for indirect immunostaining with the rabbit antiserum against-Cdc10/ankyrin repeats of Notch1 (1:1000 dilution), or the pre-immune rabbit serum (1:1000 dilution) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulins (Cappel). The samples were observed using Radiance Plus confocal laser microscope (Bio-Rad).

3. Results

3.1. Rearrangement of the Notch1 gene in a DBA/2 mouse T lymphoma cell line

Fifteen T cell lines established from spontaneously developed lymphomas of DBA/2 mice were screened for rearrangement of *Notch* genes by Southern blotting. Although no rearrangement of *Notch2, 3* or *4* was detected (data not shown), rearrangement of the *Notch1* gene was found in a cell line named DL-3 (Fig. 1). In addition to the 2-kb *Pst*I and the 4.2-kb *Bam*HI fragments derived from the unaffected *Notch1* allele, the *Notch1* middle probe hybridized with an additional 1.2-kb *Pst*I and an additional 2.7-kb *Bam*HI fragment in the DL-3 genomic DNA. To further characterize this *Notch1* gene rearrangement, we constructed restriction maps of the unaffected *Notch1* allele (in normal liver cells) and the rearranged *Notch1* allele in DL-3 (data not shown). A comparison of these two maps indicated that DL-3 has a rearrangement in one allele of the *Notch1* gene: an insert of 9 kb in the region between the Notch/lin-12 repeats and the transmembrane domain encoding sequences.

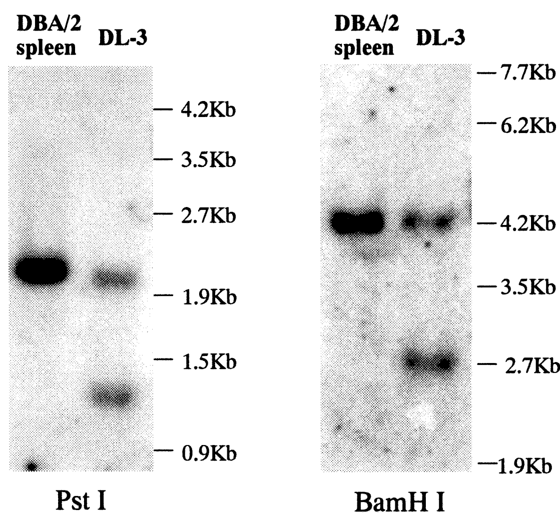

Fig. 1. Southern blot hybridization demonstrating the rearrangement of the *Notch1* locus in DL-3. Genomic DNAs from the DL-3 and normal DBA/2 spleen were digested with *Pst*I (left panel) or *Bam*HI (right panel), Southern blotted, and hybridized with the *Notch1* middle probe. Numbers on the right indicate the migration positions of DNA molecular weight markers.

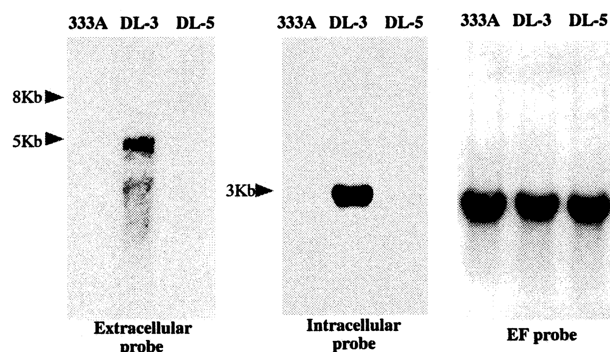

Fig. 2. The truncated *Notch1* transcripts are highly expressed in DL-3. Total RNAs from lymphoma cell lines with *Notch1* rearrangement (DL-3) or not (333A and DL-5) were subjected to Northern analyses with the *Notch1* extracellular probe (left panel) or the intracellular probe (middle panel). Approximate sizes of the full-length transcript (8 kb) and the two truncated *Notch1* transcripts (5 kb and 3 kb in the left and the middle panels, respectively) are indicated by arrowheads. For RNA loading controls, the blot shown in the middle panel was stripped and reprobed with the human elongation factor-1α (EF) probe (right panel).

3.2. Overexpression of truncated Notch1 transcripts in DL-3

Next, we analyzed whether the rearrangement of the *Notch1* genome affects the expression of *Notch1* transcripts in DL-3. Marked expression of a 3-kb and a 5-kb transcript was detected with the intracellular (Fig. 2, middle panel) and the extracellular (Fig. 2, left panel) probes, respectively, in DL-3. However, none of these unusual *Notch1* RNA species was detected in 333A and DL-5 cell lines, which have normal *Notch1* alleles. Therefore, rearrangement of the *Notch1* locus in DL-3 induced the expression of the 3-kb and the 5-kb RNA species, which only encode the intracellular and the extracellular domain of Notch1, respectively. Expression of the normal *Notch1* RNA species of 8 kb was undetectable in all of these cell lines (Fig. 2, left panel).

3.3. Structure of the truncated Notch1 transcript

To further characterize the mechanism of *Notch1* rearrangement and to define the structure of the truncated *Notch1* transcripts, a cDNA library of the DL-3 cell line was screened with the intracellular *Notch1* probe. In this study, the cDNA clones only encoding the intracellular domain of Notch1 were screened, because this type of truncated Notch protein is known to be constitutively active in Notch signaling. Among the 20 clones selected, a clone named dl-3B was found to have the longest cDNA insert, 3 kb. Therefore the complete nucleotide sequence of the cDNA insert of this clone was determined and its regions of interest are shown in Fig. 3A. Sequence analysis revealed that the 5′ terminus of the cDNA has good homology to the long terminal repeat (*LTR*) sequences of a provirus of MuLV, a highly leukemogenic type C retrovirus[22], while the remainder of the cDNA represents *Notch1* sequences which encode the transmembrane and intracellular domains of the Notch1 protein [2]. Further detailed Southern analysis with *Notch1*- and *MuLV*-specific probes (data not shown) revealed that the full-length MuLV provirus (9 kb) is inserted in the middle of an exon that encodes amino acid sequences located between the Notch/lin-12 repeats and the transmembrane domain of Notch1 (the provirus integration site corresponds to nucleotide posi-

tion 4938 of the *Notch1* cDNA, see Fig. 3B). Since the provirus had integrated in the direction of *Notch1* gene transcription, the proviral 3′ *LTR* appears to function as a promoter for the 3-kb *MuLV-Notch1* fusion transcript. On the other hand, if the *Notch1* transcription was to initiate from the normal *Notch1* promoter, it would terminate at the site of provirus integration, and the expected size of the *Notch1* transcript would be about 5 kb. In addition, the 5′ *LTR* of the integrated provirus probably provided strong enhancer(s) to the *Notch1* promoter to achieve high levels of *Notch1* transcription, and, at the same time, the sequence in the R region of the same 5′ *LTR* functioned as a termination signal for this *Notch1* transcript [24]. Thus this may be the reason why the 5-kb transcript encoding the extracellular domain of Notch1 is markedly expressed in DL-3 (Fig. 2, left panel). This 5-kb transcript may not affect the expression of *Notch1* target genes, because it lacks a region encoding the intracellular domain, which is essential for its signal transduction in the cytoplasm and nucleus.

3.4. Ectopic expression of the truncated Notch1 protein in the DL-3 cell line

Using the ATG codon at nucleotide position 5053 of the *Notch1* cDNA as an initiation methionine, the cDNA insert of the dl-3B clone can encode a protein of about 100 kDa which contains the transmembrane domain and the intracellular domain of Notch1 (Fig. 3A). Therefore expression of the Notch1 protein in DL-3, 333A, and DL-5 cell lines was analyzed by Western blotting with antibody against the RAM23 domain of Notch1 (Fig. 4A). Although none of these cell line express the 300-kDa full-length Notch1 protein, the DL-3 cell line expresses the 100-kDa truncated Notch1 protein which corresponds to the expected size of the open reading frame in the cDNA insert of dl-3B. Consistent with the nuclear transloca-

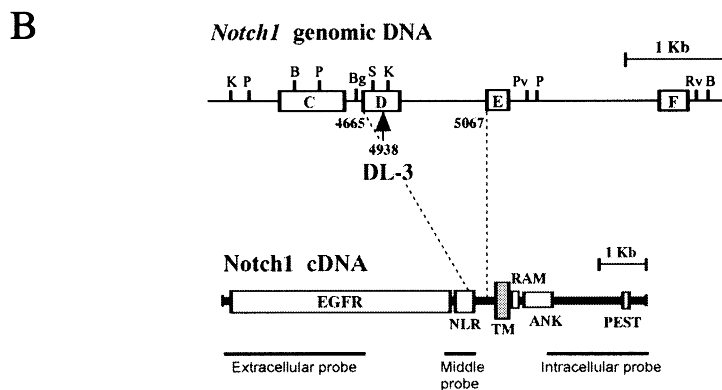

Fig. 3. Identification of a MuLV provirus insertion at the *Notch1* locus in DL-3. A: The nucleotide sequence of the 5′ terminus of the cDNA insert of the dl-3B clone is shown. Numbers in italics and roman letters indicate the nucleotide positions of the *MuLV LTR* [22] and the wild-type *Notch1* cDNA sequences [2], respectively. The ATG codon for potential initiating methionine (indicated by a bold M) locates at nucleotide position 5053 of the *Notch1* cDNA sequence. The deduced amino acid sequence appears below the nucleotide sequence. R and U5 regions in the *MuLV LTR*, and exon D and E regions of the *Notch1* genome (see below) are indicated by right-angled arrows. B: Rearranged regions of the *Notch1* genome are shown together with a schematic representation of the full-length *Notch1* cDNA. A restriction map of the rearranged region of the *Notch1* genome is shown. Exons and introns are indicated by open boxes and solid lines, respectively. Exons are labeled C, D, E and F. Numbers indicate the nucleotide positions of the *Notch1* cDNA. The insertion site of DL-3 is indicated by a vertical arrow. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; Rv, *Eco*RV; S, *Sac*I; X, *Xba*I. The structural features of *Notch1* cDNA are shown: EGFR (EGF-like repeats), NLR (Notch/lin-12 repeats), TM (transmembrane domain), RAM (RAM23-homologous domain), ANK (Cdc10/ankyrin repeats), PEST (PEST sequence motif). The extracellular, middle and intracellular probes used in Southern and Northern analyses are shown with bars.

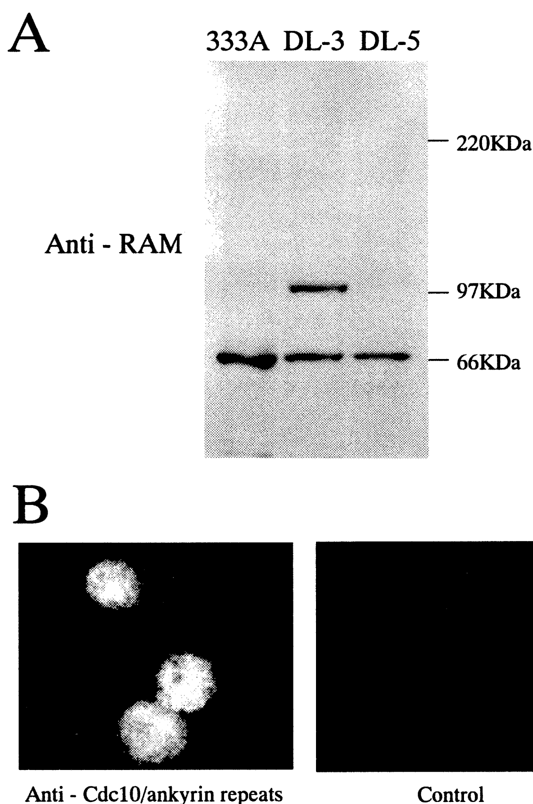

Fig. 4. Expression and subcellular localization of the truncated Notch1 protein in DL-3. A: Western analysis. Total cell lysates of DL-3, 333A and DL-5 were analyzed by Western blotting with anti-RAM23 antibody. The positions of the molecular weight markers are indicated on the right. Intense 66-kDa bands present in all lanes are non-specific signals unrelated to Notch1. B: Immunostaining. DL-3 cells stained with rabbit anti-Cdc10/ankyrin repeat serum and control pre-immune serum are shown in the left and right panels, respectively.

tion model for Notch1 signaling, immunostaining with anti-Cdc10/ankyrin repeat antibody (the anti-RAM23 antibody is not good for immunostaining) revealed that the truncated Notch1 localizes mainly in the nucleus in DL-3 cells (Fig. 4B, left panel).

3.5. Increase of HES-1 expression in the DL-3 cell line

The *HES* gene is known to be a target of Notch1 signaling. Therefore levels of *HES* mRNA were compared between DL-3 and DL-5 cell lines, with or without *Notch1* gene rearrangement, respectively. Northern blotting demonstrated that DL-3 expresses six-fold higher levels of *HES-1* mRNA (1.7 kb) than DL-5 (Fig. 5). On the other hand, we did not detect any expression of *HES-2, -3* and *-5* in these cell lines (data not shown). These results indicate that the truncated Notch1 protein expressed in the DL-3 cell line activates the Notch1 signaling pathway and thus might contribute to the development of this lymphoma in vivo.

4. Discussion

A number of studies in *Drosophila* and vertebrates have indicated that loss of the extracellular domain of *Notch* represents a gain-of-function mutation which is implicated in tumorigenesis. However, the molecular mechanisms by which these constitutively active Notch proteins induce cell transformation are unknown. The HES-1 protein, a mammalian homolog of the *Drosophila hairy and enhancer of split*, is known to function in neurogenesis, retinal differentiation and T cell lineage decisions [12,13,19]. Recently, several in vitro experiments have been reported demonstrating a link between Notch1 signaling and the activation of *HES-1* transcription; expression of the intracellular region of Notch1 together with RBP-J activates the transcription from the *HES-1* promoter carrying the RBP-J binding motif [3,8]. In HeLa cells expressing Notch1, a *HES-1*-derived promoter construct is transactivated and levels of endogenous *HES-1* mRNA are elevated in response to Delta-1, a Notch1 ligand [10]. In addition, overexpression of HES-1 leads to the silencer site-dependent repression of the CD4 promoter as well as the downregulation of endogenous CD4 expression in $CD4^{+}CD8$ helper T cells, indicating a role for HES-1 in T cell differentiation [25]. In this view, the present study is the first to report a spontaneously developed mouse T cell lymphoma cell line demonstrating a correlation between activation of Notch1 signaling and elevation of HES-1 mRNA. In the primary lymphoma samples from which the DL-3 cell line was established, we confirmed the same *Notch1* rearrangement and expression of both 3- and 5-kb truncated *Notch1* mRNA, as in DL-3, suggesting the clonality of this lymphoma and the participation of overexpressed HES-1 in this leukemogenesis. Beside *HES-1* induction, the involvement of the nuclear factor (NF) κB family of transcription factors in Notch1 signaling has been suggested; Wang et al. [26] have reported that the truncated constitutively active Notch1 has the functional properties of an Iκ-B-like regulator with specificity for the NF-κB p50 subunit and thus modulates the expression of NF-κB target genes in T cells. Furthermore, RBP-J binding motifs were found to overlap with κB elements (NF-κB binding motif) in promoters of various NF-κB-inducible genes, indicating a novel role for RBP-J in silencing NF-κB-inducible transcription [27]. Anyway, the availability of a T cell line exhibiting a Notch1 gain-of-function mutation may be instrumental in developing assays for Notch1 signaling in T cells. Finally, overexpression of the truncated Notch1 in 32D myeloid progenitors inhibits granulocytic differentiation and permits expansion of undifferentiated cells [28], suggesting that an activated Notch1 has a similar function in neoplastic cell proliferation and in other hematopoietic systems.

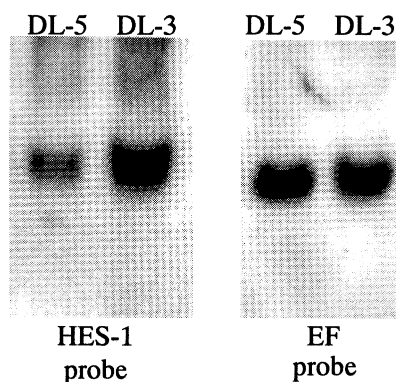

Fig. 5. Elevated expression of *HES-1* mRNA in DL-3. Total RNA isolated from DL-5, DL-3 cell lines was subjected to Northern blot analysis with the *HES-1* probe (left panel). The right panel is a blot with the EF probe for RNA loading control.

Acknowledgements: We thank Dr. R. Kageyama for plasmid pSV2-CMVHES-1, and Dr. S. Nagata for pPAN7, the plasmid containing human elongation factor-1α cDNA. This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan to S.Y.

References

[1] Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995) Science 268, 225–232.
[2] Franco del Amo, F., Gendron-Maguire, M., Swiatek, P.J., Jenkins, N.A., Copeland, N.G. and Gridley, T. (1993) Genomics 15, 259–264.
[3] Kato, H., Sakai, T., Tamura, K., Minoguchi, S., Shirayoshi, Y., Hamada, Y., Tsujimoto, Y. and Honjo, T. (1996) FEBS Lett. 395, 221–224.
[4] Kidd, S., Lieber, T. and Young, M.W. (1998) Genes Dev. 12, 3728–3740.
[5] Schroeter, E.H., Kisslinger, J.A. and Kopan, R. (1998) Nature 393, 382–386.
[6] Chan, Y.-M. and Jan, Y.N. (1998) Cell 94, 423–426.
[7] Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H. and Honjo, T. (1999) J. Biol. Chem. 274, 7238–7244.
[8] Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R. and Israel, A. (1995) Nature 377, 355–358.
[9] Kopan, R., Schroeter, E.H., Weintraub, H. and Nye, J.S. (1996) Proc. Natl. Acad. Sci. USA 93, 1683–1688.
[10] Jarriault, S., Le Bail, O., Hirsinger, E., Pourquie, O., Logeat, F., Strong, C.F., Brou, C., Seidah, N.G. and Israel, A. (1998) Mol. Cell Biol. 18, 7423–7431.
[11] Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-Okazaki, S., Tamura, K. and Honjo, T. (1997) Development 124, 4133–4141.
[12] Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992) Genes Dev. 6, 2620–2634.
[13] Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S. and Kageyama, R. (1994) J. Biol. Chem. 269, 5150–5156.
[14] Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D. and Sklar, J. (1991) Cell 66, 649–661.
[15] Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C.D., Simard, C., Kozak, C.A. and Jolicoeur, P. (1996) Genes Dev. 10, 1930–1944.
[16] Rohn, J.L., Lauring, A.S., Linenberger, M.L. and Overbaugh, J. (1996) J. Virol. 70, 8071–8080.
[17] Pear, W.S., Aster, J.C., Scott, M.L., Hasserjian, R.P., Soffer, B., Sklar, J. and Baltimore, D. (1996) J. Exp. Med. 183, 2283–2291.
[18] Gallahan, D. and Callahan, R. (1997) Oncogene 14, 1883–1890.
[19] Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G. and Salmon, P. (1996) Cell 87, 483–492.
[20] Yanagawa, S., Murakami, A. and Tanaka, H. (1990) J. Virol. 64, 2474–2483.
[21] Yanagawa, S., Kakimi, K., Tanaka, H., Murakami, A., Nakagawa, Y., Kubo, Y., Yamada, Y., Hiai, H., Kuribayashi, K., Masuda, T. and Ishimoto, A. (1993) J. Virol. 67, 112–118.
[22] Rassart, E., Shang, M., Boie, Y. and Jolicoeur, P. (1986) J. Virol. 58, 96–106.
[23] Yanagawa, S., Van Leeuwen, F., Wodarz, A., Klingesmith, J. and Nusse, R. (1995) Genes Dev. 9, 1087–1097.
[24] Jonkers, J. and Berns, A. (1996) Biochim. Biophys. Acta 1287, 29–57.
[25] Kim, H.K. and Siu, G. (1998) Mol. Cell. Biol. 18, 7166–7175.
[26] Guan, E., Wang, J., Laborda, J., Norcross, M., Baeuerle, P.A. and Hoffman, T. (1996) J. Exp. Med. 183, 2025–2032.
[27] Oswald, F., Liptay, S., Adler, G. and Schmid, R. (1998) Mol. Cell. Biol. 18, 2077–2088.
[28] Milner, L.A., Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I.D. and Martin, D.I.K. (1996) Proc. Natl. Acad. Sci. USA 93, 13014–13019.